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(54) **Transfer of mRNA using polycationic compounds**

(57) The invention provides a method for the transfer of RNA, especially mRNA, into cells and into organisms. This method is especially suited for applications in the field of gene therapy. The RNA is transferred in the form of a complex between at least one RNA and at least one cationic, especially polycationic, peptide or protein. Preferably protamine, poly-L-lysine and/or histone is used as cationic peptide or protein.

a i.p. 10^5 Hela-K^b RNA transfected
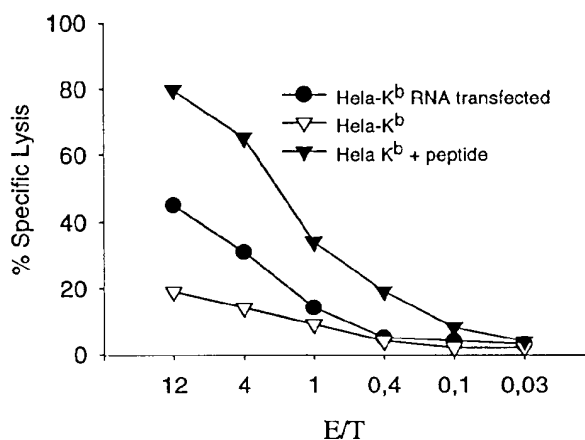
Fig. 1

Description

[0001]   The invention relates to a method for the transfer of mRNA, particularly into cells. Furthermore, the invention relates to a complex and a composition useful for performing especially the inventive method.

[0002]   A lot of methods have been developed in the past decades in order to transfer nucleic acids into cells and especially into organisms. In particular, transfer methods for eukaryotic cells were taken into account in the last years. In this connection methods which are suitable in the context of gene therapy are of particular interest.

[0003]   Gene therapy is a medical procedure that treats a disorder by replacing or counteracting a faulty gene. Such a therapy demands a high measure of safety precautions to minimize risks and dangers for the treated person.

[0004]   The common approach for the research in the field of gene therapy is the use of DNA to introduce the needed genetic information into the cell. There are several methods known which are useful and comparatively effective in delivering DNA into the cell, e.g. calcium phosphate transfection, Polybren transfection, protoplast fusion, electroporation, microinjection or lipofection. Especially lipofection has been proved to be a useful tool.

[0005]   One method especially applied in the field of gene therapy is the use of DNA-viruses as vehicles for the DNA. Viruses have the important advantage that they are infective by themselves. Therefore, they have no problems in entering the cell. On the other hand, by working with viruses a certain risk always remains. It is not possible to exclude that the virus spreads uncontrollably in the organism, in spite of the fact that obviously the used viruses have been genetically engineered to exclude this risk as far as possible.

[0006]   Usually, the DNA delivered into the cell integrates to a certain extent into the genome of the transfected cell. This could be a desired effect because this could possibly enable a long-persisting effect of the introduced DNA. On the other side, integration into the genome involves one main risk of gene therapy. By integration the introduced DNA could cause an insertion in an intact gene of the transfected cell, i.e. a mutation which disturbes the function of that particular gene. Hereby, an important enzyme, for example, could be knocked out, and the cell possibly looses its viability. Since the integration into the genome is a statistic event only a portion of the cells will die due to the integration of the delivered DNA causing lethal insertion mutations. Therefore, lethal insertion mutations will not lead to extreme drastic after-effects for the organism, in general. It is more fatal that due to the mutation caused by the insertion of the DNA the starting point for the development of a cancer could be given. For example, by destroying the gene encoding a regulatory factor for proliferation the cell can show unlimited division. Such an abnormal proliferative cell could entail a cancerous disease with all known bad consequences for the concerned patient. For this it could be sufficient that only one single cell is converted into a cancerous cell which is the starting point for the cancer development.

[0007]   When working with DNA, it is mandatory to connect the gene to be introduced with a strong promoter, e.g. the viral CMV-Promotor, in order to achieve a sufficient translation rate. The potential risks of such promoters are not exactly known, but it is not possible to exclude the insertion of said promoters in the genome of the treated cell with fatal consequences for the regulation of gene expression.

[0008]   Another dangerous aspect of using DNA in gene therapy is the induction of pathogenic anti-DNA antibodies within the treated organism causing a fatal immune defence.

[0009]   Due to these reasons in the past several considerations and attempts were made in order to circumvent the risks of DNA, especially of uncontrollable insertion of DNA in the field of gene therapy. The solution of this problem would be the use of RNA instead of DNA as a carrier of genetic information.

[0010]   RNA by itself is not able to integrate into the genome. Therefore, by the use of RNA mutations caused by insertion will not take place. One further advantage of the use of RNA is that RNA could be directly translated by the translation machinery of the cell. The step of transcription of DNA into mRNA as it is needed when using DNA for transfection is not necessary. Therefore, the genetic information is directly translated into the desired gene product, e. g. an enzyme, without delay. An appearance of anti-RNA antibodies, which could cause problems in respect to clinical applications, were not observed until now.

[0011]   In order to ensure that the RNA is identified and correctly translated within the transfected cell it is necessary to work with RNA compatible with the eukaryotic translation system. By transfecting messenger RNA (mRNA) which contains a 5'-cap and a polyA-tail and possibly further appropriate translation signals like e.g. a ribosomal binding site it is secured, that the genetic information is correctly used by the cell.

[0012]   An important applicability of RNA is the antisense approach. Here a RNA complementary to the target mRNA is transferred into the cell. Due to complemetarity both RNAs interact with each other and thereby the translation of the target mRNA is inhibited. By this technology the expression of a special undesired protein can be suppressed.

[0013]   Another important field of application of transfer of RNA are the so-called ribozymes. Ribozymes are RNA molecules with enzymatic activity which attack desired targets in the cell very specifically.

[0014]   In summary, RNA would be a very efficient tool in the field of gene therapy and in the field of molecular research on the whole.

[0015]   So far RNA was considered to be very problematical in laboratory and clinical handling. Since RNA is very prone to hydrolyses by ubiquitous ribonucleases it is not stable in solution. Even smallest contaminations of ribonu-

cleases are sufficient to degrade RNA completely. Therefore, gene therapy approaches involving nucleic acids have until now focused on DNA which is much easier in handling (Ref. 1).

[0016] Nevertheless, it has been shown that naked RNA injected into mouse skeletal muscle does lead to gene expression in vivo (Ref. 2) and that cytotoxic T cells can be primed in vivo with liposome encapsulated mRNA which so far could be prepared by an extremly complicated procedure (Ref. 3).

[0017] Recently, it has been shown by Ying et al. that a self-replicating RNA vaccine is a potentially useful candidate for the treatment of patients with cancer (Ref. 23). Vaccination is based on introducing an antigen, in this case the genetic information (DNA or RNA) for an antigen, into the organism, especially into the cell. The genetic information is translated into the antigen, i.e. into a certain peptide or protein and thereby an immune response directed against this peptide or protein is stimulated. In respect to the treatment of cancer this can be achieved by introducing the genetic information of cancer antigens, e.g. proteins solely expressed by cancerous cells. Then, these cancer antigens are expressed in the organism and an immune response is elicited which is efficiently directed against the cancerous cells eliminating them. Due to the extreme sensitivity and the very low stability of RNA the immunogenicity of this nucleic acid is normally very low. The authors of the above-mentioned article enhanced the immunogenicity of the nucleic acid by making it "self-replicating". This was accomplished by using a gene encoding RNA replicase polyprotein derived from the Semliki forest virus in combination with a RNA encoding for a model antigen.

[0018] The main disadvantage of the approach of Ying et al. could be that the viral enzyme RNA replicase is necessary to enhance the efficacy of the RNA introduced. The effects of this foreign enzyme in the organism treated are not known. This enzyme is able to replicate RNA unspecifically, i.e. it replicates RNA from viruses which could enter the organism by normal infections at random, thereby increasing the risk of dangerous infections drastically. This makes the approach of Ying et al. risky for clinical applications.

[0019] Another disadvantage when using replicase is that cells transfected with this enzyme show apoptosis, i.e. cell death, within about 24 h. These short-living cells are not able to produce the desired gene product in sufficient amounts. That is another reason why this approach is not suited for applications in the field of gene therapy where relatively long-persisting expression of the target gene has to be achieved.

[0020] Therefore, the invention has the object to provide an efficient method for introducing mRNA into cells, especially into (living) organisms which circumvents the above-mentioned problems and which is suited for therapeutical and clinical applications. This object is solved by a method showing the features of claim 1. Special embodiments of the inventive method are depicted in the dependent claims 2 to 10. A complex comprising inter alia mRNA, a composition, and the use of the complex or the composition in accordance with the inventive method are claimed in claims 11 to 25. The wording of all claims is hereby made to the content of the specification by reference.

[0021] The invention is based on the surprising results of the inventors that mRNA could be efficiently transferred into cells if the mRNA is associated or bound to a cationic peptide or protein. The use of protamine as a polycationic nucleic acid binding protein is especially effective. Use of other cationic peptides or proteins like poly-L-lysine or histone is also possible.

[0022] Protamines are basic (cationic) small proteins of molecular weights of about 4,200 Da. They are found tightly associated with DNAs in fish spermatozoa. Nearly two-thirds of the amino acid residues in protamines are basic, and these basic residues are usually found clustered, four or five in a sequence.

[0023] In the Sixties and Seventies the effect of basic proteins like protamine on the infectivity of viruses was investigated. It was shown that basic proteins provoked a sensitization of cells in respect to infectivity of viral RNA (Refs 21 and 22). In 1978 it was shown that protamine forms a complex with double stranded tRNA (Ref. 9). These research results were not considered to be important for the field of gene therapy.

[0024] The inventors now clearly showed the protective effect of basic proteins like protamine on the critical stability of single stranded RNA, especially mRNA. Based on these results a method for the transfer of mRNA into cells was developed, wherein the mRNA is transferred in the form of a complex or a comparable associated structure between at least one mRNA and at least one cationic, especially polycationic peptide or protein. Complex formation by essentially ionic interactions has the decisive effect of stabilizing the mRNA and preventing it from degradation. Thus, the genetic information is delivered into the cell without the drastic loss of efficacy which normally makes the transfer of functional RNA into cells impossible.

[0025] In general, all cationic, especially polycationic, peptides or proteins should be suited for this inventive method. In particular, nucleic acid binding peptides or proteins show good results. In a preferred embodiment the cationic/basic peptides are short and possess a length of up to 100 amino acids. Nevertheless, the invention comprises use of peptides or proteins which are larger. Usable peptides or proteins are preferably provided by purification of naturally occuring or recombinantly expressed peptides or proteins, since such substances are commonly available. Another possibility is to produce the substances by chemical synthesis. Especially suited are protamine, poly-L-lysine and histone.

[0026] Side-effects of these proteins or peptides, especially regarding gene therapy, are negligible, since the above mentioned peptides or proteins are not immunogenic. Therefore, the treatment of a patient with said peptides or proteins will not evoke an immune response against the administered composition comprising mRNA and the cationic peptide

or protein. Thus, the inventive method is excellently suited for therapeutical and clinical application. Obviously, the inventive method provides also an efficient tool in all domains of molecular research.

[0027] The tolerance of the inventive method in the field of e.g. gene therapy is a main advantage of the invention. Therefore, it is preferred to perform the inventive method in vivo, especially in clinical applications. The complex comprising mRNA and the cationic peptide or protein is preferably administered into subarachnoid spaces, peripheral lymphatic nodes, tumour tissues and/or cartilaginous tissues. Especially promising results were obtained by administration into cartilaginous tissues. In one embodiment of the invention the complex is administered into aural tissues, especially into the ear pinna. The applicability of the inventive method in aural tissues is of particular importance since conventional methods, like liposome-mediated nucleic acid transfer into aural tissues is not possible.

[0028] For delivering the complexed mRNA into the cell, especially into the organism, e.g. animals or humans, there are several possibilities. In preferred embodiments of the inventive method the complex is delivered by injection, particularly by intradermal, intramuscular, intravenous, subcutaneous and/or intranasal injection.

[0029] Advantageously, the mRNA to be transferred encodes at least one antigen. Subsequent to transfer of the nucleic acid the encoded antigen is translated by the translation machinery of the transfected cell. This antigen evokes an immune response within the organism. This response is usable to treat a certain disease, for example to treat tumours or cancer. A specific protein or peptide of the target cells to be eliminated is chosen as antigen. Another possibility is to deliver the genetic information for more than one antigen into the cell. Preferably, collections of mRNAs are transferred in order to achieve a strong immune response. Clearly, the invention comprises the transfer of genetic information encoding e.g. enzymes or other peptides or proteins or collections thereof. Furthermore, the invention comprises the delivery of antisense RNA or collections thereof into the cell.

[0030] In a preferred embodiment of the invention the collection of mRNAs is achieved by preparing a RNA library derived from e.g. tumour tissue. The collection of mRNAs could be built up by fractionated or unfractionated RNA libraries.

[0031] In an especially preferred embodiment of the invention the aim of introducing mRNA encoding antigens into the cell is to stimulate cytotoxic T cells for example. This is achieved by the presentation of antigenic peptides in connection with MHC class I complex on the surface of antigen presenting cells, which have been transfected by mRNA. This way of evoking an immune response is similar to the stimulation by viral pathogens. Simultaneously, a humoral immune response is achieved by stimulation of T helper cells due to the immunization with mRNA. Thereby, the immunization with mRNA leads to the possibility of treatment of viral infections up to the treatment of cancer. Especially the treatment of cancer is performable by immunization with an unfractionated and/or fractionated RNA library produced from tumour tissue. This is possible even in the case of very small sample amounts of tumour tissue, since it is possible to generate a cDNA library which can be amplificated and stored, if needed so. From this cDNA library RNA can be transcribed in vitro in high amounts which can be used for vaccination in the inventive manner.

[0032] In an especially preferred embodiment of the invention the mRNA comprises sequences, especially at the 5'- and/or 3'- terminal end, which are not translated within the cell. These additional sequences are especially preferred in order to achieve a further stabilization of the mRNA. Particularly in clinical applications such a further stabilization of RNA could be desirable.

[0033] For example in order to increase the translational efficacy of the transferred mRNA it could be advantageous to use a mRNA which comprises at least one internal ribosomal entry site (IRES). Such a sequence promotes the entry and binding of the mRNA into the ribosome whereby the translation rate is increased.

[0034] In a preferred embodiment of the invention the transferred RNA comprises two or more genes, e.g. a gene encoding for an antigen and a gene encoding for a factor, e.g. a cytokine, which stimulates the immune response or costimulating surface receptors. With respect to such multifunctional RNAs IRES could be used as well-suited components when constructing the corresponding vectors.

[0035] In comparison with the transfection using DNA it was shown by the inventors that the transfer of RNA using the inventive method is much more effective. About 1 μg RNA per 30 g living weight was shown to be effective in evoking a specific cytotoxic T cell response in mouse whereas 50 μg DNA per 30 g living weight were necessary to evoke the same effect. Thus, the inventive method has the considerable advantage that much less genetic material is needed to obtain the desired effect thereby reducing the costs.

[0036] Additionally the invention comprises a kit suitable for performing the inventive method. The kit comprises at least one basic peptide or protein, especially protamine or poly-L-lysine, appropriate buffer solutions and preferably RNA, especially mRNA, in dried state. In another embodiment of the kit RNA is excluded in order to enable the user to use the inventive kit for the own particular task of interest.

[0037] Furthermore, the invention comprises a complex which is formed by at least one cationic peptide or protein and at least one mRNA. Regarding the features of this complex reference is made to the above description. The inventive complex is especially useful for transfer of mRNA into cells, especially into organisms.

[0038] In a preferred embodiment of the invention at least one inventive complex as described above is part of a composition. Further ingredients of the composition are preferably buffer solutions and/or further components which

promote the stability of the mRNA. Preferably the composition comprises at least one RNase inhibitor. RNase inhibitors are commercially available and are widely used in laboratory practice. One example of a suitable RNase inhibitor is the RNase inhibitor of human placenta. It is also possible to stabilize the RNA by other treatments, e.g. special salt conditions, as they are known by experts in the art.

[0039] Furthermore, the composition could comprise at least one pharmaceutically acceptable carrier in order to provide a pharmaceutical composition useful in therapeutical applications, for example.

[0040] The invention comprises the use of the inventive complex as outlined above for the manufacturing of pharmaceutical compositions. The manufacturing of the pharmaceutical composition is performed according to standard procedures. Due to the stability of precipitated RNA it is possible to store RNA in dried state. Prior to the transfer of the RNA according to the invention the RNA e. g. is solved into an appropriate solution additionally comprising at least the basic peptide or protein and preferably further comprising stabilizing components, e.g. at least one RNase inhibitor.

[0041] Such pharmaceutical compositions are especially useful for the treatment of diseases, which are preferably caused by at least one genetic defect. Examples of such kind of diseases are congenital immunodeficiency disorders, like Cystic Fibrosis or Adenosine Deaminase Deficiency (ADA). In such cases the invention is used to deliver the correct genetic information which is absent or malfunctional in the concerned patient. If it is appropriate, the invention is used to suppress a malfunctional gene by antisense technology.

[0042] In another preferred embodiment of the invention the complex is used for the manufacturing of a pharmaceutical composition for the stimulation of an immune response. As outlined above, the inventive method can be used to introduce the genetic information for an antigen into the cell evoking an immune response directed against the certain antigen. For example, in the treatment of cancer this could be a very efficient strategy to overcome the disease. Also in the field of infections like AIDS, Hepatitis or Malaria, the use of the inventive method, especially the use of a complex for the manufacturing of pharmaceutical composition, promises good results.

[0043] Finally, the invention comprises the use of the inventive method in order to express factors, especially peptides or proteins, preferably peptides or proteins which are expressed on cell surfaces by means of which the cell is labelled. This aspect of the invention could be used to promote desired cell-to-cell interactions, for example, or to provide a target for specific destroying strategies, e.g. in the field of tumour treatment. Furthermore, the invention could be used in order to express cytokines which could e.g. stimulate the immune system and/or strengthen the immune response. Examples of such cytokines are GM-CSF, IL-2 or IFN-gamma. It is also possible to express surface receptors which increase presentation of antigens, e.g. molecules of the B7 class or surface molecules involved in cell-cell adhesion like ICAM-1, ICAM-2 and LFA-3, in particular with respect to tumor therapy and/or viral infections.

[0044] One example of the use of the inventive method is the treatment of muscular dystrophy, comprising the steps of introducing a therapeutic amount of a composition comprising a mRNA operatively encoding for dystrophin in a pharmaceutically acceptable injectable carrier in vivo into the muscle tissue whereby the mRNA is taken up into the cells and dystrophin is produced in vivo.

[0045] Further examples of diseases which could be treated by the inventive method are insulin deficiencies (transfer of mRNA encoding insulin), Phenylketonuria (transfer of mRNA encoding phenylalanine hydroxylase), Hypercholesterolemia (transfer of mRNA encoding a factor associated with cholesterol homeostasis) and Cystic Fibrosis.

[0046] The features as described and further features of the invention read from the following description of an example in conjunction with the sub-claims, whereby each of the individual features is claimed individually or in combination with each other.

Examples

[0047] In figures 1 to 4, to which reference is made in the following description, the features as here listed are shown.

Figure 1 Induction of β-Gal specific CTL (cytotoxic T lymphocytes) activity. B6 mice were immunized i.p. (intraperitoneal) with 10^5 Hela K^b cells electroporated with $\beta glacZ\beta g\alpha_n$ RNA. Spleen cells were stimulated in vitro with the synthetic peptide ICPMYARV corresponding to the amino acid sequence 497-504 of E. coli β-galactosidase. CTL activity was determined in a standard ^{51}Cr-release assay 5 d after in vitro restimulation. The targets were Hela K^b cells, Hela K^b cells transfected with $\beta glacZ\beta g\alpha_n$ RNA and Hela K^b cells incubated with synthetic peptide ICPMYARV.

Figure 2 RNA degradation protection assay. Arrowhead indicates 0.5 μg of naked $\beta glacZ\beta g\alpha_n$ RNA or protamine-condensed $\beta glacZ\beta g\alpha_n$ RNA, respectively. RNA was incubated with 2.5 % fetal bovine serum in vaccination buffer at room temperature. At the indicated time, samples were withdrawn, treated with RNase inhibitor, frozen at -70 °C and analyzed on a 1 % agarose gel.

Figure 3 Induction of β-Gal specific CTL activity. BALB/c mice were immunized as described with Unifectin-encap-

sulated protamine-condensed RNA or with protamine condensed RNA. 30 µg of protamine-βglacZβgα_n RNA + Unifectin was injected intravenously (a) or subcutaneously (b) and 30 µg of protamine-βgfpβgα_n RNA + Unifectin was injected intravenously as a control (c). Using the ear pinna (i.e.), 30 µg of protamine-βglacZβgα_n RNA (d), 1 µg of βglacZβgα_n protamine-RNA (e), 30 µg of protamine-βgfpβgα_n RNA as a control (f) and 30 µg of P13.I protamine-library-RNA (g), respectively, were injected once. Spleen cells were stimulated twice in vitro with the synthetic peptide TPHPARIGL corresponding to the H2^d epitope E. coli β-Gal (amino acid 876-884 sequence). CTL activity was determined in a standard ^{51}Cr-release assay 5 days after in vitro restimulation. The targets were P13.1 cells (H-2^d lacZ transfected) and P815 cells-(H-2^d).

Figure 4 Induction of β-Gal specific CTL activity. BALB/c mice were immunized i.e. with 30 µg of unprotected βgla-cZβgα_n RNA (a) or with 30 µg of pCMVβ DNA plasmid (b) as described in Ref. 10. Control mice were immunized i.e. with 30 µg of unprotected βgfpβgα_n RNA (c) or with 30 µg of RNase treated βglacZβgα_n RNA (d). Spleen cells were stimulated twice in vitro with the synthetic peptide TPHPARIGL. CTL activity was determined in a standard ^{51}Cr-release assay 5 days after in vitro restimulation. The targets were P13.1 cells (H-2^d lacZ transfected) and P815 cells (H-2^d). The humoral IgG anti-β-Gal immune response was detected by ELISA from sera two weeks after immunization (e).
Average values were estimated over the OD$_{490}$ Of 1:100 to 1:800 diluted sera. Mice were immunized once i.e. with 30 µg of pCMVβ DNA plasmid, 30 µg of βggfpβgα_n RNA, 30 µg of unprotected βglacZβgα_n RNA or 30 µg of protamine-βglacZβgα_n RNA.

Methods and results

Plasmids and RNA synthesis

[0048]   Capped βglacZβgα_n RNA transcripts for vaccination containing Xenopus laevis β-globin sequences were prepared by in vitro transcription with Sp6 Cap-Scribe (Boehringer Mannheim) according to the manufacturer's directions from the linearized SpjC-βglacZβgα_n, plasmid (a HindIII-BamHI fragment containing the lacZ gene was excised from pCHIIO plasmid (Pharmacia) and inserted into the HindIII and BglII sites of the pSpjC-I plasmid, which was kindly provided by J. Lord, Warwick, UK). For a negative control βggfpβgα_n RNA from the linearized pT7TS-βggfpβgα_n plasmid was prepared containing the gfp gene from Aequoria victoria with T7 Cap-Scribe (Boehringer Mannheim; the Smal-SpeI fragment of the gfp gene was excised from pCFP (Clontech) and inserted into the EcoRV and SpeI sites of pT7TS plasmid, which was generously provided by P. A. Krieg, Austin, Texas, USA). P13.1 library RNA transcripts were prepared from a linearized cDNA library from P13.I cells with T3 Cap-Scribe (Boehringer Mannheim). The cDNA library was constructed from P13.I cells using the ZAP-cDNA Synthesis Kit (Stratagene) according to the manufacturer's instructions. Prior to in vitro transcription, all linearized plasmids were extracted twice with phenolchloroform and precipitated with ethanol/sodium acetate.
[0049]   RNA transcripts were treated with DNase (2 U/µg of DNA-template, Boehringer Mannheim) at 37 °C for 15 min and extracted with phenol/chloroform. Finally RNA was precipitated with ethanol/sodium acetate, solubilized in DEPC-treated water and stored at -80 °C until use.

Immunizations with nucleic acids

[0050]   Protamine (free base, grade IV from salmon) and the protamine derivates (protamine phosphate and protamine sulphate) were purchased from Sigma. The cationic liposome Unifectin (structure see below) was obtained from A. Surovoy, Tubingen.
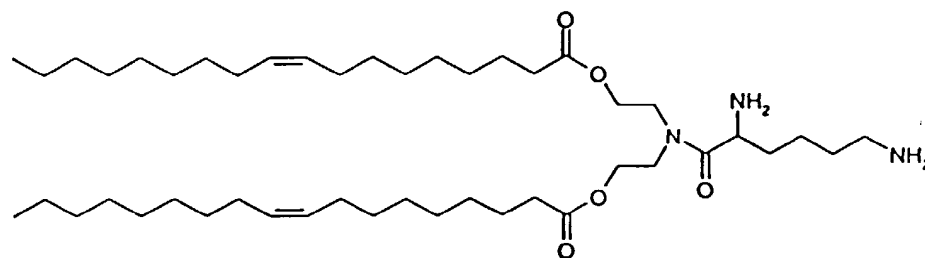
Unifectin

[0051]   A protamine : RNA ratio of 1:2 throughout was incubated in 100 μl vaccination buffer (150 mM NaCl, 10 mM HEPES, pH 7.4) at room temperature for 5 min. Unifectin-encapsulated RNA was prepared in a liposome : protamine : RNA ratio of 2:1:1, whereas Unifectin was added 10 min after incubation of protamine-RNA in 60 μl vaccination buffer at room temperature. 100 μl vaccination solution was injected i.p. (intraperitoneal), i.v. (intravenous), s.c. (subcutaneous) and i.m. (intramuscular). Naked RNA and DNA plasmid controls were prepared in 100 μl DEPC treated vaccination buffer and applied immediately. 50 μl vaccination solution was injected into each ear pinna of anaesthetized mice (Metofane, Janssen).

ELISA

[0052]   Blood samples were obtained from the tail vein of mice. Sera were prepared and ELISA plates (Greiner) were coated overnight with β-galactosidase (Sigma) in PBS buffer at 4 °C.

[0053]   After washing with 0.05 % Tween-20/PBS the plates were blocked with 1 % bovine serum albumin/PBS for 1 h at 37 °C. Sera were diluted in 0.05% Tween-20/PBS and administered to the plates for 1 h at 37°C. After further washing steps, bound antibodies were spotted with a peroxidase-conjugated goat anti-mouse IgG immunoglobulin (Sigma). The plates were washed and developed with 5-aminosalicylic acid (Sigma) in phosphate buffer and read at 490 nm.

Cell culture and ^{51}Cr release assay

[0054]   P 815 and P13.1 cells (Ref. 18) were cultured in α-MEM containing 10 % FCS, 2-ME, L-glutamine, and antibiotics. 7-9 d after immunization of mice, recipient spleens were removed and the splenocytes were restimulated with synthetic β-Gal peptide TPHPARIGL (ICPMYARV for the Hela-K[b] approach) at 50-100 mM. CTL (cytotoxic T lymphocyte) lines were generated by weekly restimulation with syngeneic, irradiated spleen cells and synthetic peptides as described in Ref. 19. Lysis of target cells was tested in a standard 5 h ^{51}Cr-release assay described previously in Ref. 20. Where applicable, target cells were pulsed with peptide during the ^{51}Cr labelling period. After incubation of effector and target cells in round-bottom 96-well plates for 5 h, 50 μl of 200 μl culture supernatant were removed and radioactivity was measured in a microplate format scintillation counter (1450 Microbeta Plus), using solid phase scintillation (Luma Plate-96, Packard). Percent specific release was determined from the amount of ^{51}Cr released into the medium (A), corrected for spontaneous release (B), and compared to the total ^{51}Cr content of the 1% Triton-X-IOO lysed target cells (C): % Specific lysis = IOO(A-B)/(C-B).

RNA degradation protection assay

[0055]   0.5 μg of βglacZβgα_n RNA was incubated for 5 min in 5 μl vaccination buffer containing 0.25 μg of protamine. At time point 0.5 μl vaccination buffer containing 5 % fetal bovine serum was added and incubated at room temperature. At an indicated period of time, samples were withdrawn and mixed with 2 μl vaccination buffer containing 10 U of RNase inhibitor (Sigma) and frozen at -70 °C. 4 μl DEPC-treated vaccination buffer containing 10 U of proteinase K and 4 μl DEPC-treated vaccination buffer containing 20% SDS and loading dye was added. Samples were analyzed on a 1% agarose gel in DEPC-treated Tris-acetate-EDTA buffer.

[0056]   To study the efficiency of RNA-based vaccines a plasmid vector for in vitro transcription of lacZ-RNA (βglacZβgα_n RNA) was engineered containing a cap structure, a poly(A) tail and flanked at the 5' end and the 3' end by untranslated regions (UTRs) from β-globin of Xenopus laevis to enhance cytosolic protein expression (Refs 4 and 5).

[0057]   To access its immunogenic potential, Hela-K[b] cells (Ref 6) were electroporated with βglacZβg_n RNA (as follows: settings for Gene Pulser (Bio-Rad): Voltage: 850 V, Capacitance: 50 μF, Current: 50 mA, Resistance:∞ Ω.

Cells (0.5 ml) were placed on ice for 5 minutes and transferred to 9.5 ml of complete α-MEM medium and incubated at 37°C over night) and injected i.p. into B6 (H2^b) mice. A specific CTL response against β-Gal after stimulation of spleen cells in vitro with the synthetic peptide ICPMYARV corresponding to the K^b presented epitope of E. coli β-galactosidase (amino acid sequence 497-504) (Ref. 7) was observed that was comparable to CTL induction upon immunization with in vitro RNA transfected dendritic cells (Ref. 8) (Fig. 1).

[0058] For direct injection of RNA into mice, protamine was used to condense and protect RNA from RNase degradation. Protamine is a small naturally occurring, arginine rich protein (Ref. 9). As shown in Figure 2, the protamine RNA complex is less sensitive to RNase activity in 2.5 % fetal bovine serum (undegraded full length RNA was visible after 60 min incubation) as compared to naked RNA, which was degraded within minutes.

[0059] To generate a specific CTL response in vivo, 30 µg of protamine-condensed βglacZβgα$_n$ RNA was encapsulated with a cationic liposome named Unifectin by a very simple procedure and injected intravenously and subcutaneously into BALB/c mice. A specific CTL response against β-Gal (Fig 3a, b) was obtained after stimulation in vitro with P13.1 cells (H2^d, lacZ transfected). Control mice were immunized intravenously with 30 µg of Unifectin-encapsulated protamine condensed βggfpβgα$_n$ RNA, encoding the gfp gene from Aequoria victoria (Fig. 3c). Intramuscular or intraperitoneal injection of liposome encapsulated RNA failed to raise specific CTL (data not shown). These results emphasize the importance of choosing the most suitable injection site for successful nucleic acid vaccination.

[0060] The efficiency of RNA vaccination using the ear pinna (i.e.) as injection site was investigated. Injection into the ear pinna of Unifectin-encapsulated protamine-condensed βglacZβgα$_n$ RNA did not generate specific CTL (data not shown), whereas a single injection of 30 µg protamine-condensed βglacZβgα$_n$ RNA without liposome triggered a specific CTL response that was recalled after stimulation in vitro using a synthetic peptide corresponding to the H2^d β-Gal epitope TPHPARIGL (amino acid sequence 876-884) (Ref. 11) (Fig. 3d). Only 1 µg of protamine-condensed βglacZβgα$_n$ RNA (a low amount of nucleic acid compared to the regular protocol for DNA vaccination in mice that involve at least 50 µg of DNA (Refs 12 and 13)) sufficed for in vivo CTL priming (Fig. 3e). Control mice immunized i. e. with 30 µg of protamine condensed βggfpβgα$_n$ RNA did not develop anti-β-Gal CTL (Fig. 3f). After demonstration that it is possible to raise CTL in vivo directed against a single protein encoded by a homogenous RNA population, the assumption was explored that a complex mRNA mixture could trigger antigen-specific CTL as well. It has previously been shown that RNA transcribed in vitro from a cDNA library can be taken up by dendritic cells (DC) in vitro and that RNA-loaded DC induce CTL responses in vivo (Ref. 8). The possibility of triggering a CTL response directed against β-Gal by employing a complex mixture of RNA was evaluated using RNA transcribed in vitro from a cDNA library of P13.1 cells (H2^d, lacZ transfected). Immunization (i.e.) with protamine-condensed, P13.1 library-RNA, for example led to a specific CTL response against β-Gal (Fig. 3g). This is remarkable, since only a low amount of of lacZ encoding RNA can be expected in the 30 µg of total unfractionated P13.1 RNA.

[0061] To test whether protection of RNA by protamine is essential for in vivo CTL priming BALB/c mice were immunized i.e. with 30 µg of unprotected βglacZβgα$_n$ RNA immediately after preparation (Fig. 4a). Unexpectedly, naked RNA immunization also generated a β-Gal specific CTL response, that was even stronger than the injection i.e. of 30 µg of naked pCMVβ plasmid DNA, for example (Fig. 4b). On the contrary, injection of unprotected βggfpβgα$_n$ RNA, as well as RNase-treated βglacZβgα$_n$ RNA did not induce β-Gal specific CTL (Fig. 4c, Fig. 4d). Furthermore the injection of unprotected βglacZβgα$_n$ RNA stored for 1 h at room temperature in buffer that was not especially made RNase-free did not induce CTL (data not shown). The development of humoral immunity was tested by β-Gal ELISA plate assay using sera 2 weeks after immunization. IgG antibodies specific for β-Gal are detectable in sera of mice immunized i. e. either with 30 µg of pCMVβ plasmid DNA, 30 µg of unprotected βglacZβgα$_n$ RNA, or 30 µg of protamine-condensed βglacZβgα$_n$ RNA. Sera of mice injected i.e. with βggfpβgα$_n$ RNA was used as control (Fig. 4e).

[0062] These results allow conclusions for vaccine development that are wholly unexpected, especially with respect to therapeutic vaccination against cancer. Although the surprising efficiency of freshly prepared naked RNA for vaccination is unlikely to promote its use in a clinical setting, protamine-protected RNA appears to have all the advantages of DNA vaccination, but without the intrinsic risk of DNA integration into the genome and the induction of pathogenic anti-DNA antibodies (Ref. 14). Tumor antigens that are already known could be used as multi-epitopic RNA vaccines without regard for HLA expression in the vaccinee (as is required for peptide vaccines (Ref. 15)). Moreover, since heterogenous RNA populations lead to an antigen-specific CTL response, RNA can be prepared from a cDNA library established from a small tumor sample (Ref. 8). This approach seems to present a way to induce immunity against certain types of cancer in patients in which tumor rejection antigens have not been identified, although it bears the potential risk of inducing autoimmunity, as does any other complex, tumor-derived vaccine, such as gp96 (Ref. 16).

[0063] Immunization into the ear pinna was highly efficient. Ear pinna tissue was tested 24 h after injection with protamine-condensed βglacZβgα$_n$ RNA for expression of the lacZ gene by X-gal staining. The intensity of β-gal expression was strong and not distinguishable from that of a control pCMV-β DNA plasmid (data not shown). In addition, the reason for the expression of RNA which occurs so readily in an immunogenic way, needs to be elucidated (RT-PCR confirmed that βglacZβgα$_n$ RNA was present in the ear pinna for more than 48 h after injection (data not shown)). One possibility is that RNA transduces dendritic cells directly in vivo. It is certainly an advantage that the the in vivo uptake

of RNA is mediated without the need for any adjuvants or transfection vehicles such as cationic liposomes, which often cause latent toxicity in higher doses. Cationic liposomes are highly toxic toward macrophages and induce a drop in the production of at least two immunomodulators by activated macrophages (Ref. 17). The RNA-protective protamine does not appear to be immunogenic since no protamine-specific IgG antibodies were detected in sera of mice (data not shown). For practical purpose, RNA can be stored in precipitated form and solubilized in transfection buffer containing protamine directly before vaccination.

[0064]   The results of the invention can be summarized as follows. To study the efficiency of RNA based vaccines, RNA coding for the model antigen β-galactosidase (β-Gal) was transcribed in vitro from a lacZ gene flanked by stabilizing Xenopus laevis β-globin 5' and 3' sequences and was protected from RNase degradation by condensation with the polycationic peptide protamine. The liposome-encapsulated condensed RNA-peptide complex, the condensed RNA-peptide complex without liposome or naked, unprotected RNA, was injected into BALB/c (H2^d) mice. All preparations led to protein expression in the local tissue, activation of H-2-restricted anti-β-Gal cytotoxic T lymphocytes (CTL) and production of IgG antibodies reactive against β-Gal. RNA-triggered CTL were even more efficient in the lysis of lacZ transfected target cells as CTL triggered by a lacZ DNA eukaryotic expression vector. Immunization with RNA transcribed from a cDNA library from the β-gal expressing cell line P13.1 again led to β-Gal specific CTL and IgG induction. Thus, both naked and especially protected RNA can be used to elicit a specific immune response, whereby the protected RNA is stable in vitro for a longer period of time. RNA vaccines can be produced in high amounts and have the same major advantages as DNA vaccines but lack the potentially harmful effect of DNA integration into the genome.

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[0065]

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Claims

1. Method for the transfer of mRNA, particularly into cells, characterized in that the mRNA is transferred in the form of a complex between at least one mRNA and at least one cationic, especially polycationic, peptide or protein.

2. Method according to claim 1, characterized in that the peptide or protein is a nucleic acid binding peptide or protein, particularly protamine, poly-L-lysine and/or a histone, wherein protamine is preferred.

3. Method according to claim 1 or claim 2, characterized in that the method is performed in vivo, wherein preferably the complex is administered into subarachnoid spaces, peripheral lymphatic nodes, tumour tissues and/or cartilaginous tissues, wherein cartilaginous tissues are further preferred.

4. Method according to one of claims 1 to 3, characterized in that the complex is administered into aural tissues, especially into an ear pinna.

5. Method according to one of the preceding claims, characterized in that the complex is administered by injection, particularly by intradermal, intramuscular, intravenous, subcutaneous and/or intranasal injection.

6. Method according to one of the preceding claims, characterized in that the mRNA encodes at least one antigen.

7. Method according to one of the preceding claims, characterized in that the mRNA is a collection of mRNAs.

8. Method according to one of the preceding claims, characterized in that the mRNA comprises sequences, especially at the 5'- and/or the 3'-terminal end, which are essentially not translated during expression.

9. Method according to one of the preceding claims, characterized in that the mRNA comprises at least one internal ribosomal entry site.

10. Method according to one of the preceding claims, characterized in that the mRNA is further stabilized by at least one factor, especially a RNase inhibitor.

11. Complex comprising at least one mRNA and at least one cationic, especially polycationic, peptide or protein.

12. Complex according to claim 11, characterized in that the peptide or protein is a nucleic acid binding peptide or protein, particularly protamine, poly-L-lysine and/or a histone, wherein protamine is preferred.

13. Complex according to claim 11 or claim 12, characterized in that the mRNA encodes at least one antigen.

14. Complex according to one of claims 11 to 13, characterized in that the mRNA comprises sequences, especially at the 5'- and/or the 3'-terminal end, which are essentially not translatable during expression.

15. Complex according to one of claims 11 to 14, characterized in that the mRNA comprises at least one internal ribosomal entry site.

16. Complex according to one of claims 11 to 15, characterized in that the mRNA is a collection of mRNAs.

17. Composition comprising at least one complex according to one of claims 11 to 16.

18. Composition according to claim 17, characterized in that it comprises at least one factor in order to further stabilize the mRNA.

19. Composition according to claim 17 or claim 18, characterized in that it is a vaccine.

20. Composition according to one of claims 17 to 19, characterized in that it is a pharmaceutical composition comprising the at least one complex in an effective amount and preferably at least one pharmaceutically acceptable carrier.

21. Use of a complex according to one of claims 11 to 16 for the manufacturing of a pharmaceutical composition for the treatment of diseases, especially diseases due to genetic defects and/or viral infections.

22. Use of a complex according to one of claims 11 to 16 for the manufacturing of a pharmaceutical composition for the stimulation of an immune response.

23. Use according to claim 21 or claim 22, characterized in that the pharmaceutical composition is to be administered into subarachnoid spaces, peripheral lymphatic nodes, tumour tissues and/or cartilaginous tissues, wherein cartilaginous tissues are preferred.

24. Use according to one of claims 21 to 23, characterized in that the pharmaceutical composition is administered into aural tissues, especially into an ear pinna.

25. Use according to one of claims 21 to 24, characterized in that the pharmaceutical composition is to be administered by injection, particularly by intradermal, intramuscular, intravenous, subcutaneous and/or intranasal injection.

a   i.p. 10⁵ Hela-K^b RNA transfected

Fig. 1

Fig. 2

a 30 µg protamine
ßglacZßgα RNA
Unifectin i.v.

b 30 µg protamine
ßglacZßgα RNA
Unifectin s.c.

c 30 µg protamine
ßggfpßgα RNA
Unifectin i.v.

d 30 µg protamine
ßglacZßgα RNA i.e.

e 1 µg protamine
ßglacZßgα RNA i.e.

f 30 µg protamine
ßggfpßgα RNA i.e.

g 1 µg protamine
P13.1 library RNA i.e.

Fig. 3

a 30 µg ßglacZßgα RNA i.e.   b 30 µg pCMVß
DNA plasmid i.e.   c 30 µg ßggfpßgα RNA i.e.

d 30 µg ßglacZßgα RNA
RNase treated i.e.   e IgG Antibodies 2 weeks
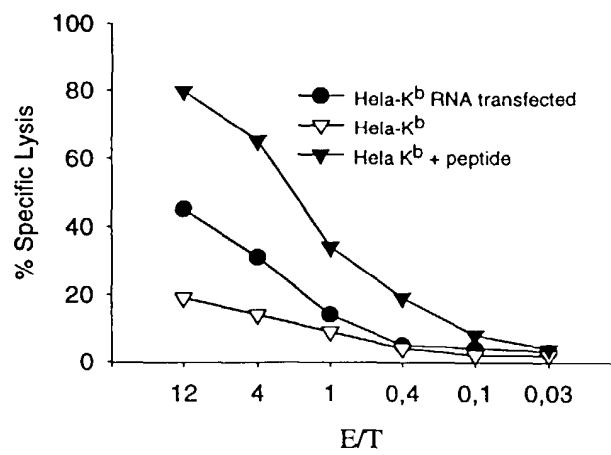
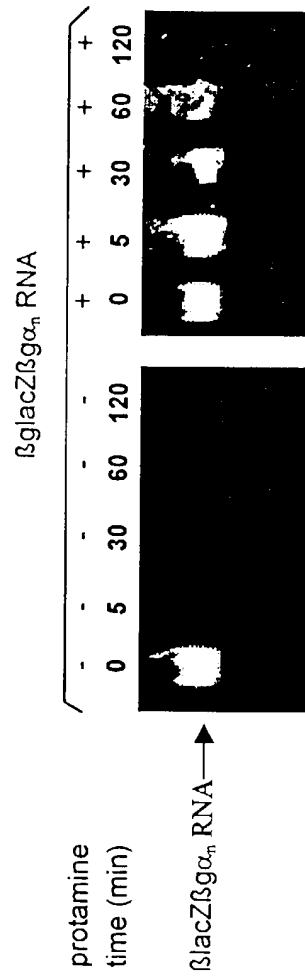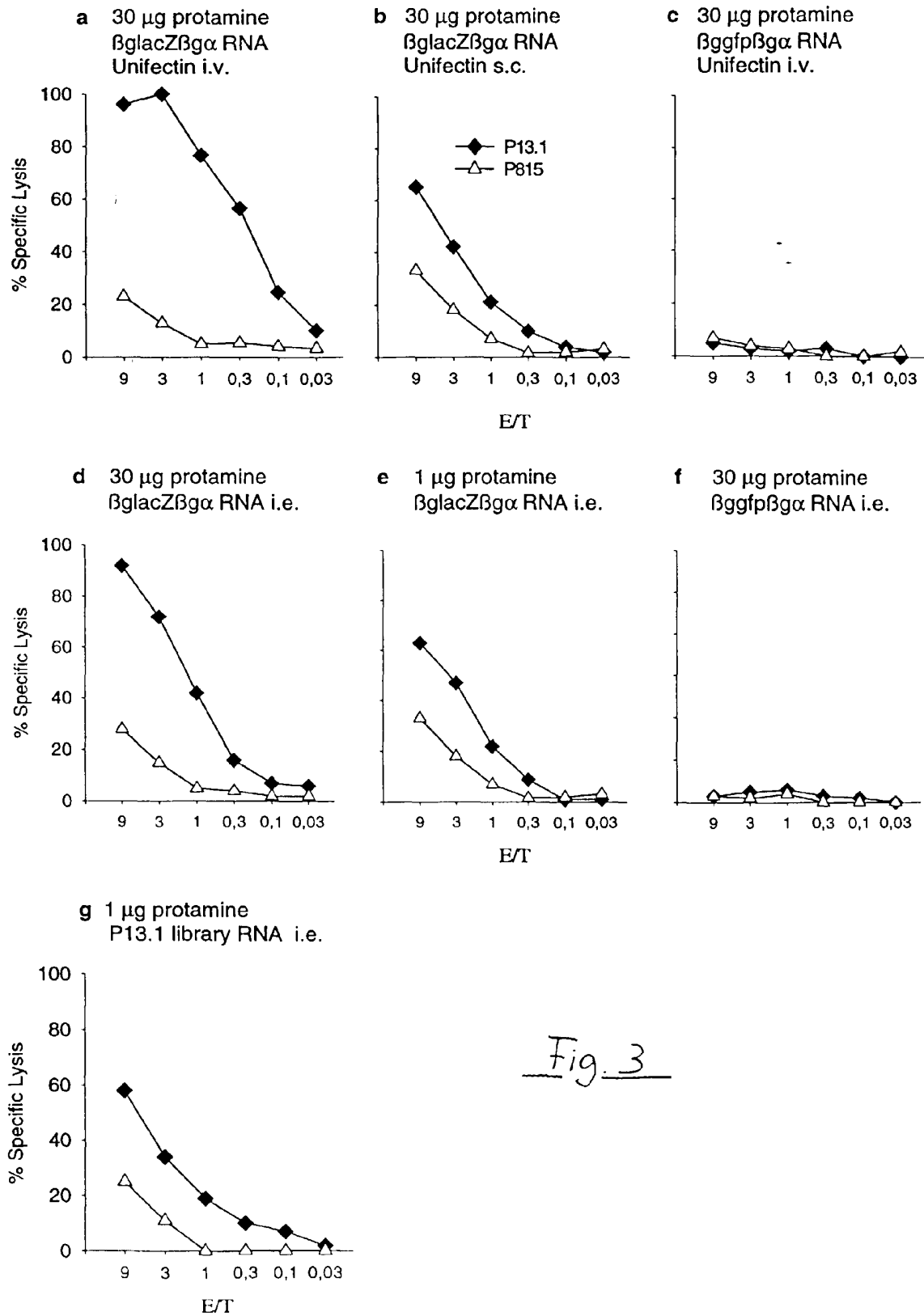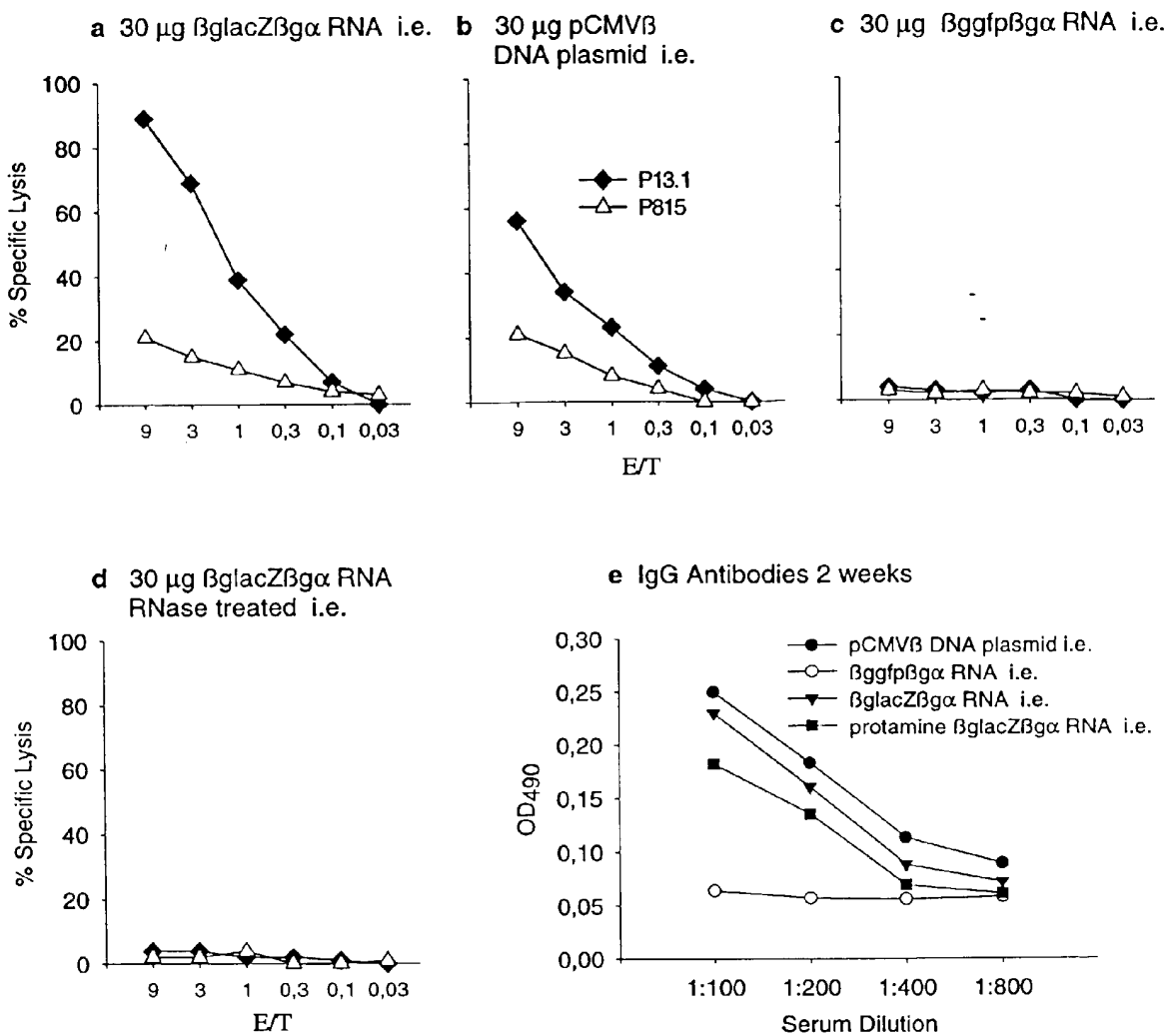*Fig. 4*

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number

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proceedings, as the European search report

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	US 5 945 400 A (SCHWARTZ BERTRAND ET AL) 31 August 1999 (1999-08-31)	1,2,5-7, 11-13, 16,17, 19-22,25	C12N15/87 A61K48/00 A61K38/00 A61P31/12
Y	* column 2, line 60 - column 3, line 10 * * column 8, line 27-39 * * column 10, line 37-45 * * claims 1-5, 27 *	1-6, 8-15, 17-25	
X	FISHER K J ET AL: "The transmembrane domain of diphtheria toxin improves molecular conjugate gene transfer." BIOCHEMICAL JOURNAL, vol. 321, no. 1, 1 January 1997 (1997-01-01), pages 49-58, XP000867796	1,2,7,8, 10-12, 16-18	
Y	* page 51, Transfections * * figure 7 * * page 55, paragraphs 2,3 *	3-6,8,9, 13-15, 19-22,25	
	-/--		TECHNICAL FIELDS SEARCHED (Int.Cl.7) C12N A61K A61P

INCOMPLETE SEARCH

The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

see sheet C

Place of search	Date of completion of the search	Examiner
BERLIN	7 February 2000	ALCONADA RODRIG.., A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
O : non-written disclosure
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

**INCOMPLETE SEARCH
SHEET C**

Although claims 1, 2, 6-10, as far as concerning an in vivo method, and
claims 3-5 are directed to a method of treatment of the human/animal body
(Article 52(4) EPC), the search has been carried out and based on the
alleged effects of the compound/composition.

PARTIAL EUROPEAN SEARCH REPORT

Application Number

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D,Y	FORG P ET AL: "Superiority of the ear pinna over muscle tissue as site for DNA vaccination." GENE THERAPY, vol. 5, no. 6, June 1998 (1998-06), pages 789-797, XP000867787 * figures 1-4; table 1 * * page 790-793 *	3-5, 23-25	**TECHNICAL FIELDS SEARCHED (Int.Cl.7)**
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ANNEX TO THE EUROPEAN SEARCH REPORT
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This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.
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